Commentary

Inhibition of Death Receptor Signaling by FLICE-inhibitory Protein as a Mechanism for Immune Escape of Tumors

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Cell death by apoptosis is a tightly regulated physiological process that enables the elimination of unwanted cells. It is crucial for embryonic development and the maintenance of tissue homeostasis, but also for defense against certain infectious diseases and cancer.

Apoptosis can be triggered from outside the cell, generally after cell–cell contact, by a family of transmembrane proteins called death receptors, which belong to the TNF family of receptors. Six human death receptors (Fas [Apo-1, CD95], TNFR-1, TRAMP [WSL-1/Apo-3/DR-3/LARD], TNF-related apoptosis-inducing ligand [TRAIL]R-1 [DR-4], TRAILR-2 [DR-5, TRICK-2, KILLER], and DR-6) have been identified to date (1, 2), and all contain a cytoplasmic sequence named "death domain" (DD) that couples each receptor to caspase cascades essential for the induction of apoptosis. The best studied signaling pathway is the one triggered by binding of Fas ligand (L) to Fas. Schematically, multimerization or clustering of Fas upon binding of the membrane-bound form of FasL recruits the bipartite molecule FADD (Fas-associated death domain, composed of an NH_2-terminal death effector domain [DED] and a COOH-terminal DD). FADD binds to Fas (via homophilic DD–DD interactions) and recruits the upstream DED-containing caspase-8 (and probably caspase-10) to the receptor via homophilic DED–DED interactions. Caspase-8 (or -10) within this newly formed death-inducing signaling complex then proteolytically autoactivates itself and initiates apoptosis by subsequent cleavage of downstream effector caspases (caspase-3, -6, and -7) (Fig. 1).

Fas signaling is known to be implicated in peripheral deletion of autoimmune cells, activation-induced T cell death, and CTL-mediated target cell death (for review see references 1 and 3). To avoid inappropriate cell death and disease, however, death receptor signals must be tightly controlled. It is known that death receptor apoptosis can be inhibited at different points: at the receptor level (by receptor endocytosis, soluble ligands, and/or decoy receptors), during signal transduction, and at the effector stage (e.g., caspase inhibitors CrmA, p35, and inhibitor of apoptosis proteins [IAPs]). Recently, we identified a new family of viral inhibitors of death receptor–mediated cell death named vFLIPs (FADD-like IL1β–converting enzyme [FLICE]/caspase-8–inhibitory proteins) that are found in several herpesviruses (including oncogenic human herpesvirus 8/Kaposi's sarcoma–associated herpesvirus and molluscipox virus) and inhibit DED–DED interactions between FADD and caspase-8 and -10 (4). Cellular homologues of vFLIPs were subsequently identified by us and others (cFLIPs; aliases Casper, iFLICE, FLAME-1, CASH, CLARP, MRIT, and usurpin) and shown to structurally resemble caspase-8, except that they lack proteolytic activity (5, 6). Their inhibition of caspase-8 activation renders cells resistant to apoptotic signals transmitted by Fas and all other death receptors known to date (Fig. 1).

Although the exact physiological function of cFLIP has yet to be completely elucidated, a role in disease was rapidly suspected. Indeed, several viruses, some of which are oncogenic, and human malignant melanomas express high levels of FLIP (4, 5). It was therefore postulated that virally infected cells and tumor cells may thereby acquire a certain degree of immune privilege by becoming resistant to FasL and perhaps other death ligands. The in vivo relevance of this hypothesis has since remained open and uncertain, however, as CTLs can lyse their targets through both Fas- and perforin-dependent pathways, and FLIP has only been shown to inhibit the former, at least in vitro (7).

In this issue, papers by Medema et al. (8) and Djerbi et al. (9) have clarified this issue. Both clearly demonstrate in different tumor models that in vivo expression of FLIP confers an advantage to tumors within an immunocompetent setting. In addition, they show that induction of tumor cell death by death receptor triggering is a more important mechanism of defense against tumors than had been suspected to date.

Djerbi et al. (9) generated mouse A20 lymphoma transfectants that stably overexpress viral FLIP from HHV8. These vFLIP-A20 cells, when compared with mock-transfected A20 cells, were shown to be resistant to Fas-mediated apoptosis by inhibition of caspase-8 activation but also showed decreased caspase-9 and -3 activation after triggering by FasL. Additionally, when grown in the continuous presence of death stimuli (agonistic anti-Fas antibody), vFLIP selectively allowed A20 cells to grow clonally. When tested in vivo using syngeneic (BALB/c) or semiallogeneic (BALB/c × C57BL/6) F1 recipients injected subcutaneously, both the frequency of tumor appearance and the time to tumor appearance were drastically higher for

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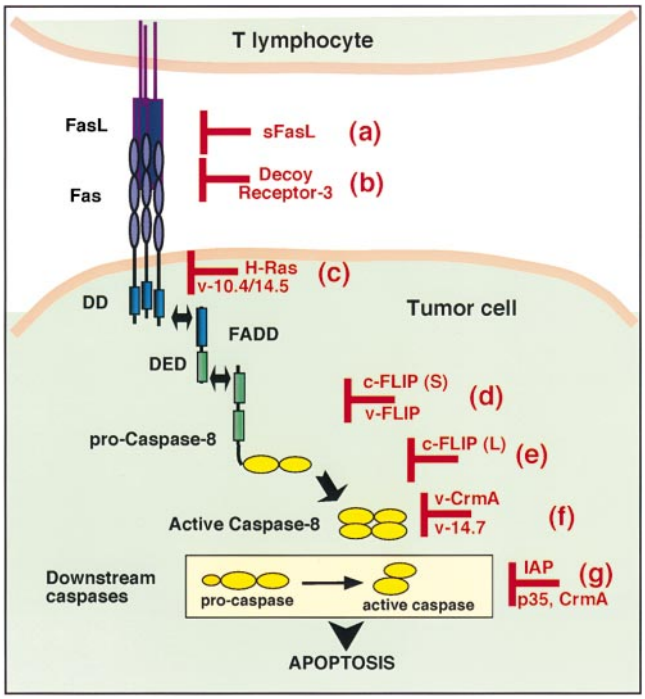

Figure 1. Fas (death receptor) signaling pathway and its inhibition. Fas (or other death receptor) signaling is triggered on target cells by receptor tri(multi)merization subsequent to FasL (or other appropriate trimeric death ligand). Subsequent recruitment of FADD (by DD–DD interaction) and procaspase-8 or -10 (by DED–DED interaction) leads to upstream caspase (caspase-8 or -10) autoactivation, which initiates apoptosis by subsequent cleavage of downstream effector caspases (caspase-3, -6, and -7). Death receptor apoptosis can be inhibited at different points (red): at the receptor level, e.g., by soluble ligands (a) and/or decoy receptors; (b) by receptor internalization, e.g., by adenovirus E3-10.4/14.5K (c); by downregulation of receptor expression, e.g., by oncogenic H-Ras (c); during signal transduction by FLIPs (d, e, and f); and at the effector stage (by caspase inhibitors, e.g., cowpox-encoded CrmA, baculovirus p35, adenovirus 14.7, and/or IAPs [g]).

vFLIP-A20 cells as compared with mock-A20 cells. Depending on the vFLIP-A20 clone tested, in syngeneic mice vFLIP-A20 cells induced tumors in roughly 90% of mice versus 32% for mock-A20 cells. Similarly, in semiallogeneic mice, vFLIP-A20 cells induced tumors in roughly 65–80% of mice, depending on the clone used, versus 17% for mock-A20 cells. Further experiments revealed that this difference is most likely due to T cell immune escape conferred by vFLIP, as tumor establishment and growth of transfected and mock A20 cells was virtually identical in SCID mice.

The experimental approach used by Medema et al. (8) is quite different to that of Djerbi et al. (9) but provides us with exciting complementary information. These authors have assessed the effect of cellular FLIP in tumorigenesis and immune escape using stable cFLIP transfectants of two distinct cell lines: (a) a Fas-transfected Moloney murine leukemia virus–induced lymphoma (MBL2-Fas) and (b) a mouse embryo cell tumor line (AR6) generated by transfection of adenovirus type 5 E1A and mutant EJ-*ras* oncogenes. The latter express low but detectable levels of Fas, whereas the former express high levels of Fas. Independent cFLIP transfectant clones from both the MBL2-Fas and AR6 cell lines were shown to be resistant to Fas-mediated cell death in vitro as compared with mock-transfected counterparts. In agreement with previous results (7), these FLIP transfectants are, however, sensitive to CTL-induced apoptosis in vitro, the perforin pathway being able to induce target cell apoptosis despite caspase-8 (and -10) inhibition by FLIP.

When these cell lines are injected in vivo, similar to the results of Djerbi et al. (9), Medema et al. (8) show that transfectants expressing little or no FLIPs are rejected in the majority of mice, whereas injection of the same number of cells expressing high amounts of FLIP consistently resulted in tumor development. When injected into nude mice, both cell types result in tumors that grow equally fast no matter how much FLIP they express. Thus, FLIP offers significant protection from the in vivo immune response to these tumors, and protection is not limited to tumors with high Fas expression (MBL2-Fas). Additionally and interestingly, when the same experiments were performed in perforin-deficient mice with the AR6 cell line, AR6-FLIP tumors grew nearly as efficiently as in wild-type mice, suggesting that elimination of this tumor by the immune system depends almost exclusively on the Fas pathway. Last but not least, when the rare tumors that had developed in mice injected with MBL2-Fas cells expressing low amounts of FLIP were analyzed in vitro, they were shown to have become Fas resistant and high expressors of FLIP. Thus, tumor cells appear to be selected in vivo for elevated FLIP expression, most likely due to selective pressure by the immune system.

The novel and complementary experimental data pre-

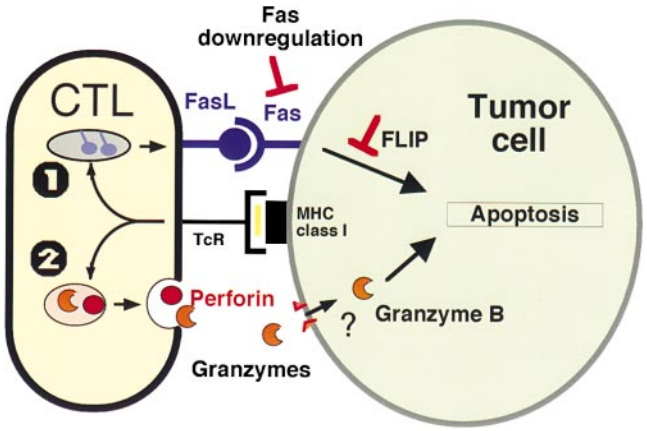

Figure 2. Inhibition of death receptor signaling as a mechanism of tumor immune escape. CTLs use two major pathways (perforin/granzyme granule exocytosis and Fas) to induce target cell (tumor) death by apoptosis after TCR–MHC–peptide complex engagement. By downregulating Fas surface expression or by producing FLIP, tumor cells can escape immune destruction mediated by the Fas death receptor signaling pathway in vivo, despite the persistence of a functional perforin/granzyme granule exocytosis pathway.

sented in the papers by Medema et al. and Djerbi et al. bring about two important new pieces of information. The first is that Fas-mediated apoptosis is a more important mechanism of defense against tumors than had been suspected to date. The second is that FLIP expression by tumors is a significant and novel mechanism of immune escape from T cell immunity in vivo (Fig. 2). This may appear to be a surprise, as FLIP does not affect perforin-mediated lysis by CTLs in vitro. However, as suggested by Medema et al. (8), this discrepancy may be due to limitations of classical in vitro cytolysis assays that may not reflect the situation occurring in the tumor microenvironment as accurately as currently conceived. In line with this observation, recent evidence suggests that to trigger the release of perforin/granzyme B from cytoplasmic granules, stronger and more persistent TCR signals may be required than for the release of FasL (Fig. 2). Furthermore, whereas partial agonistic MHC peptides are capable of eliciting FasL but not perforin cytotoxicity, strong signals from fully agonistic MHC peptides trigger both pathways (10). Consequently, in vivo CTL recognition of tumor cells as "nonself" may be inefficient, leading to preferential activation of FasL and leaving the perforin pathway virtually unimplicated.

In humans, FLIP has been shown to be overexpressed in melanomas, tumors that elicit T cell responses including the generation of melanoma-directed CTLs. Despite evidence for the in vivo generation of such CTLs, spontaneous regression of malignant melanoma only rarely occurs. The mechanisms thought to be responsible for this tumor immune escape to date include the expression of local inhibitory factors by tumor cells, such as transforming growth factor β, IL-10, and FasL, deficient antigen processing by tumor cells or loss of MHC expression, the lack of immunogenicity and costimulation for CTL activation, and defective lymphocyte homing to tumors. In certain tumor cell types, downregulation of Fas by oncogenic Ras is also observed, thereby rendering tumor cells resistant to FasL (11). Functionally, this has the same effect as overexpression of FLIP, which can now be added to the above list with reasonable confidence. Although not reported to date, FLIP upregulation may well be implicated in the pathogenesis of tumors other than melanomas.

Current attempts to improve cancer survival depend essentially on early diagnosis and the development of new treatment modalities, one of the most promising being immunotherapy. Given the new findings described herein, strategies to inhibit FLIP expression and/or FLIP-mediated inhibition of death receptor signaling may prove to be a useful complementary approach to the treatment of cancer.

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